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Original article

Identification of multiple HLA-A*0201-restricted cruzipain and FL-160 CD8$^+$ epitopes recognized by T cells from chronically *Trypanosoma cruzi*-infected patients

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Abstract

Chronic Chagas disease occurs in 16 million individuals chronically infected by the protozoan *Trypanosoma cruzi* in Latin America, and may lead to a dilated cardiomyopathy in 10–30% of patients. A vigorous cellular immune response holds parasitism in check. However, up to now, few *T. cruzi* proteins have been shown to be recognized by CD8$^+$ T cells from Chagas disease patients. In this study, we designed 94 peptides derived from *T. cruzi* proteins cruzipain and FL-160, predicted to bind to HLA-A2 molcules. After in vitro binding assays to HLA-A*0201, 26 peptides were selected, and their recognition by PBMC from Chagas disease patients was tested with the IFN-gamma ELISPOT assay. All 26 peptides were recognized by PBMC from at least one patient. Furthermore, a tetrameric HLA-A*0201 complex built with the cruzipain 60–68 peptide that was frequently recognized in the periphery also bound to CD8$^+$ T cells from a heart-infiltrating T cell line obtained from a single patient with Chagas disease cardiomyopathy. Thus, our results suggest that the recognition of CD8$^+$ T cell epitopes in cruzipain and FL-160 may have a pathogenic or protective role in chronic Chagas disease.

Keywords: CD8$^+$ T cells; Chagas disease; ELISPOT; Tetramer; Epitopes

1. Introduction

Chagas disease is caused by the intracellular protozoan parasite *Trypanosoma cruzi* and affects 16–18 million people in Latin America. Most patients survive the acute phase of the disease, remaining asymptomatic for many years. However, 10–30% of patients develop an inflammatory dilated CD8$^+$ T cell-rich cardiomyopathy (Chagas disease cardiomyopathy, CCC), with a fatal outcome in 30% of cases [1,2]. The heart inflammatory mononuclear infiltrate is known to play a pathogenic role in CCC. Because of the lack of a vaccine and effective treatment during the chronic phase, CCC is still a major public health problem in many countries.

Experiments in murine models of *T. cruzi* infection have suggested that CD8$^+$ T cells are involved in the control of infection. It has been demonstrated that mice deficient for β2-microglobulin, MHC class I or CD8$^+$ T cells showed a higher parasitemia than normal littermates during the acute phase of infection [3–6]. Furthermore, CD8$^+$ T cells are the

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predominant lymphocyte population present in the heart tissue of mice chronically infected with *T. cruzi*, and the depletion of the $CD8^+$ T cell population increases the number of parasite nests in the heart during acute infection [7]. Recently, it was shown that cytotoxic T lymphocytes (CTL) from PBMC of Chagas disease patients displayed cytotoxicity and IFN-γ production against epitopes of amastigote-stage surface protein-1 (ASP-1), ASP-2 and trypomastigote-form surface antigen-1 (TSA-1) from the trans-sialidase family of *T. cruzi* proteins [8,9]. However, there are limited data on *T. cruzi*-specific memory $CD8^+$ T cells from Chagas disease patients and no direct data showing whether heart-infiltrating $CD8^+$ T cells from CCC patients recognize *T. cruzi* epitopes.

In order to investigate the recognition of *T. cruzi* epitopes by $CD8^+$ T cells from Chagas disease patients, we have screened 94 nonamer and decamer peptides from cruzipain (CZ) [10] and FL-160 (FL) [11] predicted to bind to HLA-A2 with an in vitro HLA-A2 binding assay, given the high frequency of this HLA allele in the Brazilian population [12]. Cruzipain and FL-160 are secreted/membrane proteins from *T. cruzi* [11,13–15], with a high likelihood of being presented by the MHC class I pathway [16]. The IFN-γ ELISPOT assay was performed in PBMC from Chagas disease patients with peptides selected from those submitted to binding assays. In order to detect parasite-specific $CD8^+$ T cells in heart lesions of CCC patients, we used HLA-A*0201-cruzipain and FL-160 peptide tetrameric complexes.

2. Material and methods

2.1. Patient and control samples

Peripheral blood of 12 Chagas disease patients (10 CCC and two asymptomatic; 11 HLA-A2 positive and one HLA-A2 negative) was collected with anticoagulant (heparin for cell cultures or EDTA) for this study. Six HLA-A2-positive *T. cruzi* seronegative, healthy individuals, were used as controls. Heart-infiltrating T cells were obtained from a transvenous endomyocardial biopsy sample from the patient #11, diagnosed as severe CCC, with positive serology for *T. cruzi*. Sample collection procedures have been approved by the Internal Review Board, School of Medicine, University of São Paulo.

Chagas disease patients and healthy individuals were typed for HLA-A2 using a SSP-PCR reaction. DNA was obtained from peripheral blood samples collected with EDTA by DTAB/CTAB method as described [17]. The sequence-specific primers used in PCR reaction to HLA-A2 were 5′: TGG ATA GAG CAG GAG GGT 3′ and 5′: CAA GAG CGC AGG TCC TCT 3′.

2.2. PBMC and PBMC-derived T cell lines

Mononuclear cells from heparinized peripheral blood samples were obtained by Ficoll gradient centrifugation. Peptide-induced PBMC-derived T cell lines (5×10^6 per well) from Chagas disease patients were established with 5 μg/ml of peptide stimulus every week in the presence of 10^6 irradiated PBMC per ml as feeder cells (50 Gy) in 1 ml of Dulbecco's Modified Eagle's medium supplemented with 2 mM of L-glutamine, 1 mM sodium pyruvate, MEM's non-essential amino acids and MEM's vitamins (all from GIBCO, Grand Island, NY, USA), 50 μg/ml of gentamicin, 10 mM HEPES buffer, 10% of normal human serum (complete medium) plus IL-2 (100 U/ml, Proleukin IL-2, Chiron, USA), IL-7 (5 ng/ml, PeproTech, NJ, USA) and IL-15 (5 ng/ml, PeproTech) in a humidified, 5% of CO_2 atmosphere, in order to test the specificity of tetramer complexes.

2.3. Heart-infiltrating T cell line

Endomyocardial biopsy fragments from HLA-$A2^+$ patient #11 were minced and cultured in 96-well flat-bottom culture plates in DMEM (Gibco) with 10% of inactivated human serum, supplemented with 40 U/ml IL-2 (Hoffman-La Roche, Nutley, NJ, USA), in the presence of autologous irradiated (50 Gy) PBMC (10^5 per well) as described [18]. Lymphoblasts were later expanded by two 15-day rounds of re-stimulation with PHA (5 μg/ml), 50 Gy-irradiated PBMC (10^6 per ml) in complete medium plus 40 U/ml of IL-2. After two rounds of expansion, lymphoblasts were cultured in DMEM 10% of human serum supplemented by IL-2 (100 U/ml), IL-7 (5 ng/ml) and IL-15 (5 ng/ml) and stimulated by PHA. $CD8^+$ cells were purified using magnetic beads attached to a monoclonal antibody against human CD8 (M450, Dynal). The beads were removed using Detach-a-Bead antibodies (Dynal, Oslo). No *T. cruzi* parasite growth was observed during endomyocardial biopsy explant cultures as detected by direct visualization of the highly mobile trypomastigote forms.

2.4. Selection of synthetic peptides and binding assay

The sequences of *T. cruzi* proteins—cruzipain (A45629, gi:323055) [13] and FL-160 (JH0823, gi:542406) [11]—were scanned using a computer algorithm to identify 9- and 10-mer sequences binding to HLA-A*0201 [19,20]. Ninety-four peptides were selected and synthesized using solid-phase Fmoc with amidated C-terminals (Chiron) and analyzed by reverse-phase high performance liquid chromatography (Shimadzu, Tokyo, Japan). Peptide quality was assessed by Maldi-Tof mass spectometry (Micromass, UK). The binding affinity of the synthetic peptides to purified soluble HLA-A2.1 molcules was quantified by measuring the binding inhibition of a radiolabeled standard probe peptide as described [21]. Peptides with a high affinity for HLA-A*0201 ($IC_{50} < 500$ nM), as well as some peptides showing undetectable binding to HLA-A*0201, were selected for ELISPOT assay. These sequences were re-synthesized using solid phase technology using (Fmoc) strategy as described [22,23], and peptide quality analyzed as described above.